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# Protein Oxidation in Huntington Disease

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## Abstract

Huntington disease (HD) is an inherited neurodegenerative disorder caused by expansion of CAG repeats in the *huntingtin* gene, affecting initially the striatum and progressively the cortex. Oxidative stress, and consequent protein oxidation, has been described as important to disease progression. This review focuses on recent advances in the field, with a particular emphasis on the identified target proteins and the role that their oxidation has or might have in the pathophysiology of HD. Oxidation and the resulting inactivation and/or degradation of important proteins can explain the impairment of several metabolic pathways in HD. Oxidation of enzymes involved in ATP synthesis can account for the energy deficiency observed. Impairment of protein folding and degradation can be due to oxidation of several heat shock proteins and Valosin-containing protein. Oxidation of two enzymes involved in the vitamin B6 metabolism could result in decreased availability of pyridoxal phosphate, which is a necessary cofactor in transaminations, the kynurenine pathway and the synthesis of glutathione, GABA, dopamine and serotonin, all of which have a key role in HD pathology. In addition, protein oxidation often contributes to oxidative stress, aggravating the molecular damage inside the cell.

**Keywords:** Huntington disease, protein oxidation, oxidative stress, energy deficiency, pyridoxal 5-phosphate.

**Abbreviations:** BDNF, brain-derived neurotrophic factor; DDAH1, dimethylarginine dimethylaminohydrolase 1; DNP, dinitrophenyl; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GSH, glutathione; HD, Huntington disease; HNE, hydroxynonenal; Hsp, heat shock protein; Htt, Huntingtin; KMO, kynurenine 3-monooxygenase; KYNT, kynurenine aminotransferase; mHtt, mutant huntingtin; NMDARs, N-methyl-D-aspartate receptors; NOS, nitric oxide synthase; 3-NP, 3-nitropropionic acid; 3-NT, 3-nitrotyrosine; OXPHOS, oxidative phosphorylation; PLP, pyridoxal 5-phosphate; Prx, peroxiredoxin; QUIN, quinolinic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; TRiC, TCP-1 ring complex; VCP, valosin-containing protein; VDAC1, voltage-dependent anion channel 1.

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## Introduction

Huntington disease (HD) is a dominantly inherited neurodegenerative disorder characterized by progressive degeneration of neurons in the striatum and, to a lesser extent, in the cerebral cortex. Clinical symptoms include psychiatric and cognitive abnormality, as well as involuntary movements and postures (chorea, dyskinesia and dystonia) of the legs, trunk and face. With the progression of the disease, motor rigidity and dementia predominate. HD usually manifests in midlife, develops over 15-20 years, and inevitably leads to death. The disease was first described by George Huntington as a hereditary disease under the name of chorea (from the Greek "*choreia*", a dance, because of the involuntary movement of extremities). In 1993 it was discovered that the disorder involves an expansion of CAG trinucleotide repeats in exon 1 of the huntingtin (*htt*) gene, leading to an elongated polyglutamine sequence (polyQ) at the N-terminal of the huntingtin (Htt) protein [1]. HD appears when the number of glutamines expands beyond 40. It is known that age of onset and severity correlate with the length of expansion [2]. Pre-symptomatic patients present a significant atrophy of the caudate and putamen [3], suggesting that neurodegenerative events begin years before the occurrence of clinical symptoms. HD is accompanied by marked neuronal loss and proliferation of astrocytes expressing glial fibrillary acidic protein (GFAP) [4].

Although Htt is a widely expressed 350-kDa protein, its physiological function remains unknown. It has antiapoptotic properties, a role in vesicle trafficking and the endosome/lysosome pathways and, in cortical cells, indirectly regulates the expression of brain-derived neurotrophic factor (BDNF), a prosurvival factor for striatal neurons. It is well known that the presence of mutant Htt (mHtt) results in the formation of both intranuclear and cytoplasmic ubiquitinated aggregates containing the protease-resistant mutated N-terminal Htt fragment in neurons of affected areas. There is recent controversy about whether protein aggregation directly impairs the ubiquitin-proteasome system activity, resulting in increased production of aggregated proteins [5].

Even though aggregates often correlate with toxicity [6], polyQ proteins can also be toxic in the absence of detectable aggregates formation [7]. Recent evidences indicate that soluble oligomers can be more important than insoluble aggregates in HD pathogenesis [8-11]. Three factors are key to understanding the pathogenesis of HD: i) excitotoxicity, in which striatal neurons show increased sensitivity to glutamatergic activation by N-methyl-D-aspartate receptors (NMDARs), leading to excitotoxic neurodegeneration [12]; ii) impaired energy metabolism; and iii) oxidative stress. This review focuses on protein oxidative damage and its consequences, such as impaired energy metabolism, protein folding and pyridoxal-5 phosphate metabolism.

## **1. Oxidative stress in Huntington disease**

The first signs of oxidative stress in brain areas affected by HD were described by several research groups in the eighties and nineties. It was reported that mitochondrial dysfunction was due to a decrease in several mitochondrial enzymes involved in respiration [13,14]. A first trial with  $\alpha$ -tocopherol, which reduces oxyradical damage to cell membranes, was performed in 1995 in an attempt to slow down the course of HD [15]. Although d-alpha-tocopherol treatment had no effect on neurologic and neuropsychiatric symptoms for overall group, a significant therapeutic effect on neurologic symptoms was observed for patients early in the course of the disorder.

The evidence of oxidative stress in HD has been exponentially growing since then. Many research groups have described impairment in mitochondrial ATP generation, leading to an energy defect in humans and in several HD models [16-20]. Complex II and III of oxidative phosphorylation (OXPHOS) seem to be the most affected, with complex IV having a milder defect. Reduced activity of other enzymes involved in oxidative metabolism, such as pyruvate dehydrogenase, has also been reported in affected areas of HD patients [21]. Similarly, loss of aconitase activity was reported in the caudate and putamen in symptomatic patients with caudate/putamen atrophy [17] and in the striatum in samples obtained postmortem [22]. It

remains to be determined whether these defects are a consequence of or play a pivotal role in striatal degeneration.

Oxidative damage to DNA and lipids has also been reported to be increased in HD. The levels of 8-hydroxydeoxyguanosine (OH8dG), a product of oxidative damage, in nuclear DNA were significantly elevated in human HD [10,16] and in a mouse model [23]. Stable products of lipid peroxidation (e.g., F2-isoprostanes) are increased in HD cerebrospinal fluid [24]. In R6/1 transgenic mice that express a human mutated *htt* exon 1, evidence of striatal lipid peroxidation has also been reported [25].

In addition to reactive oxygen species (ROS), reactive nitrogen species (RNS) seems to play a role in HD [26]; increased levels of 3-nitrotyrosine (3-NT) in cortex and striatum have been reported [16]. Activation of NMDA receptors results in  $\text{Ca}^{++}$  increase, which activates the neuronal form of nitric oxide synthase (nNOS). The nitric oxide generated can react to superoxide ions to generate peroxynitrite radical ( $\text{ONOO}^-$ ). Complexes of the mitochondrial electronic chains, and other mitochondrial enzymes like aconitase, are especially vulnerable to the effects of RNS. Thus, ROS and RNS act synergistically to increase the mitochondrial defect. However, although nitrosative stress has been involved in neuroinflammation and neurodegeneration in HD, nitric oxide can also have a cytoprotective role [27].

In HD, the initial cause of increased oxidative insult is the presence of mHtt, which has been shown to increase levels of ROS in neuronal and non-neuronal cells [6]. Aggregation of a fragment of mHtt directly causes free radical production [28] and inclusion bodies may act as iron-dependent centers of oxidative stress [29]. Iron disorder described in HD would exacerbate such a stress situation [30]. Impairment of mitochondrial metabolism would increase the generation of ROS in the respiratory chain, the main intracellular source of ROS. In HD, microglia overactivation and dysregulation correlates with disease severity [31] and involves NADPH oxidase, the primary source of extracellular ROS production. Intracellular ROS have been found

to act as second messengers to amplify the proinflammatory function of microglia-mediated neurotoxicity [32]. Clearly, oxidative stress due to mHtt would result in a vicious circle aggravating the evolution of the disease.

## **2. Vulnerability of the nervous system to oxidative stress**

What makes the nervous system especially susceptible to oxidative stress? Possible explanations include i) its oxidative metabolism, with a high oxygen consumption rate and strong dependence on aerobic carbohydrate metabolism; ii) the relatively low levels of antioxidant pathways and the fact that they decrease during aging, which explains, in part, the existence of several age-related neurodegenerative diseases; iii) the high polyunsaturated fatty acids content of the membranes, which makes them more susceptible to oxidative damage [33]; and iv) the fact that neurons are postmitotic and therefore damaged macromolecules that cannot be repaired or degraded would accumulate progressively inside the cells. However, oxidative stress plays a role in many neurological diseases. Why is the striatum the main target area in HD? Although the molecular mechanism leading to HD pathogenesis remains unknown, two factors probably play a key role: excitotoxic neurodegeneration due to glutamatergic NMDARs activation [12] and increased sensitivity to oxidative stress. In the context of the present review, it is important to understand why striatum is less resistant to the oxidative burst and its consequences.

It is known that acute poisoning with mitochondrial toxins (cyanide, sodium azide, and 3-nitropropionic acid) is often associated with striatal degeneration in humans and in laboratory animals [34]. Mitochondrial defects of genetic origins (e.g., mutation or deletion of mitochondrial DNA or nuclear DNA) can lead to striatal degeneration [35]. In the conditional mouse model Tet/HD94 that expresses a mutated Htt fragment selectively in the forebrain under the tet-regulatable system [36], levels of catalase activity were decreased by 50% in the striatum, compared to cerebellum, of 20-month-old mice [37]. This supports the observation that at 20

months the mouse striatum presented lower levels of protein carbonylation compared to cortex and cerebellum. This suggests less endogenous ROS generation in the striatum, which would result in decreased antioxidant capacity. In this context, striatum in young rats has been shown to display decreased superoxide dismutase, catalase and glutathione peroxidase activities, and lower levels of glutathione (GSH) compared to cortex or other brain areas [38].

### **3. Protein oxidation in HD**

Under oxidative stress conditions, proteins undergo a series of structural modifications that in many cases result in loss of function. One well-documented protein alteration resulting from oxidative stress is carbonyl formation. It is used as a marker of protein oxidation because it is a major oxidative modification and can be easily detected and quantified by Western blot with anti-dinitrophenyl (DNP) antibodies [39-41], a technique variously referred to as oxy-blot, carbonyl immunoblot or oxyproteome (when proteins are previously separated by 2D). Protein carbonyl derivatives are the result of oxidation of some amino acid side-chains, including arginine, proline, lysine, and threonine residues. Carbonyl derivatives can also be formed by secondary reactions with reactive carbonyl compounds on lipid peroxidation products, such as hydroxynonenal (HNE), and by glycation and glycoxidation reactions [42]. Several studies published more than 10 years ago reported increased protein carbonylation in HD in comparison to control samples [16,23,43]. In addition, carbonylated proteins and oxidative stress are correlated with disease stage [44], suggesting that oxidative stress may play an important role in the pathogenesis of HD.

Despite all these observations, only a few studies have been designed specifically to identify target proteins. The first report was published by the Butterfield group using the R6/2 transgenic mice model [45]. In samples from striatum of HD patients, our laboratory extensively studied and identified carbonylated proteins in comparison to samples from control cases [22,37]. Oxidation of the mHtt protein has also been reported [11]. From these studies one can classify



the damaged proteins in several groups: those involved in energy metabolism, in folding and oxidative stress defense, in vitamin B6 metabolism, and a miscellaneous group of unrelated proteins (Table 1).

### 3.1. Proteins involved in energy metabolism

As described above, besides mHtt aggregation, energetic dysfunction was one of the first traits described in HD. Different enzymes, mainly mitochondrial ones involved in ATP generation pathways, have been described to present decreased activity in human HD samples or HD models. However, whether this is the result of decreased gene transcription, protein degradation or enzyme inactivation remains to be established. Nevertheless, several proteins from this group are known to suffer carbonyl formation, either in humans and/or the transgenic R6/2 mouse tissue samples. Three are members of the glycolytic pathway: enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase. Perluigi et al. [45] found that carbonylation of  $\alpha$  and  $\gamma$  enolase in R6/2 mice resulted in their inactivation, while our group did not find significant differences in GAPDH or pyruvate kinase activity in postmortem human samples [37].

Five mitochondrial proteins involved in energy metabolism were also carbonylated. Two are enzymes of the Krebs cycle (citrate synthase and aconitase), two are members of the OXPHOS (subunit 2 of cytochrome b-c1 -complex III- and the alpha subunit of ATP synthase), and the fifth is mitochondrial creatine kinase. Oxidation resulted in enzyme inactivation, with a remaining activity of ATP synthase and creatine kinase below 50%, compared to controls. Mitochondrial aconitase is an iron-sulfur protein that catalyzes the stereospecific isomerization of citrate to isocitrate via cis-aconitate. It shows a particular susceptibility to oxidative damage due to release of iron ions from its iron-sulfur cluster in the active center [46]. Therefore, due to the effects of iron as a superoxide generator, inactivation of aconitase can initiate a cascade with the potential to cause a dramatic increase in the cellular burden of oxidative damage.

The key role of mitochondrial impairment in HD is highlighted with two chemical models of HD using inhibitors of succinate dehydrogenase (complex II): 3-nitropropionic acid (3-NP) and malonate. Mice treated with the irreversible inhibitor 3-NP have striatal lesions, exhibit choreiform and dystonic movements, and demonstrate cognitive deficits, all similar to HD [47]. Interestingly, increased oxidative stress was evident prior to the appearance of morphological lesion, and protein oxidation in striatal synaptosomes precedes 3-NP-induced striatal lesion formation [48]. Mice treated with the reversible inhibitor malonate are also used as a chemical model [49]. Both 3-NP and malonate produce energy depletion and lesions that closely resemble those of HD.

In human HD striatum, creatine kinase activity decreased by 65% [37]. The observed carbonylation and inactivation of mitochondrial creatine kinase in human HD compared to controls was also detected in a murine model [45]. The two creatine kinase isoforms (cytosolic and mitochondrial) phosphorylate creatine; this molecule constitutes the most important energy buffering and transport system of the cell, especially in muscle and neuronal tissue [50]. Mitochondrial creatine kinase is located in the intermembrane space and the phosphocreatine produced is then transported to the cytosol [51]. Creatine therapy provides neuroprotection and delays motor symptoms in a transgenic animal model of HD [52,53], thus suggesting enhanced cerebral energy metabolism as a protective mechanism against neurodegeneration. However, this neuroprotective effect could not be replicated in clinical trials, in which the treatment period may have been too short and concentrations too low [54;55]. Nevertheless, creatine supplementation lowers glutamate levels in humans, indicating a therapeutic potential in HD [55]. The huge quantity of information obtained concerning the protective role of creatine in HD models has led to a reassessment of the possible therapeutic value of higher doses of creatine. Phase III clinical trials are ongoing to evaluate treatments with 40 g creatine/day ([clinicaltrials.gov](http://clinicaltrials.gov)).

Taken together, these results are in agreement with the profound impairment of mitochondrial bioenergetic pathways described in HD, which produces severe ATP depletion and

increased lactate levels [56,57]. In HD patients and mouse models alike, increased lactate concentrations in the cerebrospinal fluid, elevated blood glucose levels and defects in mitochondrial complexes II, III and IV have been consistently described [58]. Thus, similar to what has been described in Parkinson disease [59], oxidative modifications in HD that result in mitochondrial dysfunction will increase ROS generation and secondary excitotoxicity. In this context, and like in the case of creatine, although initial trials with the antioxidant idebenone (a synthetic analog of coenzyme Q10) did not show significant differences between groups [60], treatments with higher doses (2400 mg/day) are now in phase III clinical trials (clinicaltrials.gov).

### 3.2. Proteins involved in oxidative stress defense and protein folding

Many neurodegenerative diseases including Alzheimer, Parkinson and polyQ diseases are thought to be caused by protein misfolding. In HD, molecular chaperones are recruited by the aggregated mHtt as abnormally folded; thus mHtt may reduce the availability of HSPs, increasing protein aggregation. In this context, the protective role of molecular chaperones in HD is well known [61]. Against this backdrop, carbonylation of three chaperones has been published: Hsp90 from R6/2 mice and, from human striatum, Hsc71 (a member of the Hsp70 family) and T complex protein 1 subunit beta (a chaperonin-containing t-complex peptide 1 -TCP1-) (Table 1). Oxidation could impair their chaperone activity. As a consequence, increased polyQ oligomerization and aggregation would decrease mHtt degradation, exacerbating the symptoms. The role of the Hsp70 family has been well studied in HD. In mouse models, loss of Hsp70 aggravates pathogenesis [62], while its induction reduces changes in striatal electrical activity [63]. Moreover, Hsp70/Hsc70 can reduce polyQ toxicity and aggregation in a cellular model of HD [64]. TCP1, also called TRiC (TCP-1 ring complex), assists protein folding upon ATP hydrolysis, acting downstream of Hsp70 and Hsp40. In the HD context, TRiC involvement has been shown to prevent polyQ aggregation and may have an early role hindering Htt access to pathogenic

conformations [65]. There is much evidence that elevated levels of protein chaperones suppress polyQ protein misfolding. Heat shock transcription factor 1-activating compounds, the main regulator of the heat shock response, markedly suppress neurodegeneration in several HD models [66,67]. Thus, activation of the heat shock response appears to be an attractive therapeutic target.

In human brain affected by HD, antioxidant defense proteins were strongly induced in striatum, but also detectable in cortex [22]. Among them, peroxiredoxins (Prxs) exerted their antioxidant role in cells through their peroxidase activity whereby  $H_2O_2$ , peroxynitrite, and a wide range of organic hydroperoxides are reduced and detoxified. Prx1 and Prx6 have been described to be primarily expressed in astrocytes and microglia [68]. The presence of higher amounts is due to astrocyte proliferation and/or increased gene expression as a consequence of microglia activation. Prx1 and Prx6 are present in a 2D gel electrophoresis as two spots [22]; one of them can be hypothesized to be an oxidized form of the enzyme. This assumption is based on the fact that in lens cells under oxidative stress, Prx1 show a specific oxidation of the active site residue Cys52 to cysteic acid, which produces an acidic pI shift. [69]. Even though no specific analysis has yet been conducted, one can speculate that in HD the more basic spot detected in the Prx1 immunostain would be the unmodified form of Prx1 and the more acidic would be the oxidized form of Prx1. A similar trend is observed in Prx6, which also appeared as two spots with a similar pI shift. In fact, Prx1 and 6 are homologous proteins, and both Prxs have a highly conserved Cys at the active site (Cys44 in Prx6). Oxidation of these key residues would lead to enzyme inactivation that in turn would worsen the oxidant burst.

### 3.3 Vitamin B6 metabolism

Two enzymes, pyridoxal kinase (PDXK) and antiquitin 1 ( $\alpha$ -aminoadipic semialdehyde dehydrogenase or ALDH7A1), involved in the metabolism of pyridoxal 5-phosphate (PLP), the

active form of vitamin B6 were identified from the proteomic analysis of carbonylated proteins in human HD [37]. PDXK is responsible for pyridoxal phosphorylation, resulting in activation of vitamin B6, and can also phosphorylate both pyridoxine and pyridoxamine, which are converted to PLP through the oxidase PNPOX [70] (Fig. 1). PLP is a necessary cofactor in more than 140 distinct enzymatic activities. Whether PDXK oxidation results in enzyme inactivation is not known at present; however, in the same study [37], PDXK showed a 30% decrease in striatal HD samples compared to controls. This is not unusual, since it is known that protein oxidation labels proteins for degradation. Thus, PLP synthesis could be impaired both by the decrease in PDXK levels and by its oxidation. These results were also observed –with greater impact– in the Tet/HD94 conditional mouse model [37].

In 2005, a rare disorder was identified with neonatal seizures and severe epileptic encephalopathy due to mutations in PNPOX [71]. The seizures typically involve irregular involuntary muscle contractions (myoclonus), abnormal eye movements, and convulsions. Most untreated patients died within a year of their birth. When treated, patients were unresponsive to anticonvulsants and pyridoxine, but responsive to PLP [72]. High doses of PLP have been very efficient in controlling seizures when given orally or via a nasogastric tube.

Antiquitin 1 (ALDH7A1) acts as a D1-piperidine-6-carboxylate (P6C) dehydrogenase. The role of antiquitin 1 in PLP metabolism was known because mutations in the *ALDH7A1* gene cause pyridoxine-dependent seizures, a disease affecting newborns and children. In these cases, mutations abolish antiquitin 1 activity and the accumulating substrate -P6C- inactivates PLP by forming a Knoevenagel condensation product [73]. The lack of PLP causes seizures and is associated with learning difficulties. These seizures are fully controlled by pyridoxine [74]. Thus, antiquitin 1 oxidation observed in human HD could result in its inactivation, exacerbating the lack of PLP available as a cofactor.

From these results we hypothesized that decreased PLP levels, caused by impairment of its synthesis and inactivation by P6C, would have severe consequences in the affected areas: i) enzymes involved in the synthesis of neurotransmitters like GABA, dopamine, and serotonin require PLP for this activity; ii) all transaminases would be affected, including those found in the kynurenine pathway and those using glutamate as a substrate/product; and iii) synthesis of glutathione from homocysteine involves two PLP-dependent enzymes (Fig. 1).

In HD, striatal neurodegeneration is reflected by intrinsic neurons deficiency in the neurotransmitters, particularly in GABA. In postmortem HD studies, the concentration of this neurotransmitter in HD is reduced to less than one-third of normal values in the striatum [75], with deficits exceeding 25% in the cortex [76]. Similarly, in the R6/2 mouse model, striatum-specific lower concentrations of GABA were also found [77]. In an attempt to increase GABA-mediated neurotransmission, five patients with HD were given L-glutamate (the substrate for glutamic acid decarboxylase) and pyridoxine for 2 years; no improvement of motor and behavioral function was detected [78]. In the light of our results, pyridoxine administration would not be efficiently converted to PLP due to pyridoxal kinase deficiency. As a consequence of decreased PLP availability, glutamate decarboxylase would not be able to produce GABA from glutamate.

Glutamate decarboxylase impairment related to lack of PLP would also result in increased levels of glutamate. The excitotoxic model of HD proposes a relative excess of glutamate as the mechanism for cell death in HD, especially in the striatum. In this context, the combined glutamate and glutamine concentration has been found to be elevated in the striatum of HD patients [79]. In R6/2 mice, a profound metabolic alteration in glutamate/glutamine cycling was found in different brain areas, including the striatum [80].

In addition to glutamatergic stimulation from the cortex, the striatum is the predominant target of dopaminergic neurons that originate from the substantia nigra [81]. Lack of PLP would result in DOPA decarboxylase impairment, leading to decreased dopamine levels. Recent reports

demonstrate that impaired dopamine release in HD mice models contribute to progressive motor phenotype [82], and striatal dopamine levels are decreased in the striatum of R6/2 mice [83]. However, the relative overactivity of the dopaminergic system also seems to be a major cause of involuntary movements in HD. Thus, the pattern of cerebral dopamine abnormalities in HD is complex, evidenced by the variable clinical benefit of both dopamine antagonists [84] and agonists [85] in treating HD symptoms.

Depression is the most common psychiatric disorder in HD patients, and in the general population, serotonergic dysregulation might be related to develop depression. As shown in Fig. 1, serotonin synthesis would also be affected by PLP deficiency. Although serotonin metabolism has been little characterized in human HD, it is interesting to note that in R6/2 mice, serotonin levels are significantly decreased [83].

Lower levels of PLP would also affect GSH synthesis and, as a consequence, the entire GSH-dependent antioxidant defense system. However, published results are not conclusive. In HD patients, lower levels of reduced GSH were detected both in plasma [86] and in red blood cells [87]. Nevertheless, increased total glutathione levels (GSH+GSSG) were observed in cortical and striatal mitochondria of the R6/2 mice [88], and the physiologically relevant GSH/GSSG ratio has not been measured.

In HD, alterations in the kynurenine pathway have largely been demonstrated [89,90]. In this pathway, the enzyme kynurenine aminotransferase (KYNT), catalyzes the conversion of kynurenine to kynurenic acid, the latter having a neuroprotective effect through modulation of the mitochondrial function [91]. A reduced kynurenic acid:kynurenine ratio has been reported in HD, suggesting a decreased activity of kynurenine aminotransferase [92]. Like all aminotransferases, this enzyme is PLP-dependent, so impaired activity due to a PLP deficit would explain this decreased activity (Fig. 1). As a consequence of increased kynurenine levels, the presence of both 3-hydroxykynurenine (3-HK), a free radical generator, and quinolinic acid (QUIN), would

increase. This has been demonstrated in mouse models of HD and humans [93;94]. Intrastriatal administration of QUIN, an agonist of NMDARs, selectively affects the GABAergic neurons in a mouse model [95]. This produces a phenotype similar to that observed in human HD, and for that reason QUIN treatment is used as a chemical model of HD. In model systems either in vitro or in vivo, 3-HK and QUIN have been shown to cause neurodegeneration via a combination of excitotoxic mechanisms and oxidative stress [96]. Thus, the shift away from the synthesis of kynurenic acid and towards the formation of 3-HK and QUIN may be important in the pathology of HD. In this context, inhibition of the enzyme kynurenine 3-monooxygenase (KMO), which catalyzes conversion of kynurenine to 3-HK, would shift the flux in the pathway towards the formation of kynurenic acid. Thus, KMO has been recently used as a target of pharmacological inactivation therapy in mouse models [89;97].

### 3.4. Mutant Huntingtin

MHtt protein is cleaved into a number of polyQ-containing N-terminal fragments that misfold and form soluble monomeric and oligomeric proteins as well as insoluble aggregates [8]. Oxidative stimuli enhance the polyQ-expanded truncated N-terminal Htt, also called N171 [98]. N171 Htt interacts with and chemically reduces copper (II) [99]. Recently, it has been reported that copper binding promotes oligomerization, modulating the N-terminal Htt structure [11]. These noncovalently cross-linked oligomeric forms are finally remodeled by oxidation of Cys115 and/or Cys119, which then form intermolecular disulfides. More important, the authors showed slower clearance of oxidizable N171 Htt from the soluble cell fraction, indicating that modulation of the Htt oxidation state can alter soluble mHtt levels in HD. This is especially relevant since several publications have shown that soluble N-terminal fragments of mHtt or amyloid-like aggregates, at levels of aggregation that precede inclusion body formation, might exert toxicity [100,101]. Thus, after Cys oxidation, N-terminal fragments from mHtt are more prone to form soluble oligomeric



species that are potentially more toxic than aggregates or inclusion bodies. This idea may also apply to other neurodegenerative diseases, where there is evidence that soluble oligomers may be particularly important for toxicity [100;102].

### 3.5 Glial Fibrillary Acidic Protein

In HD, proliferation of astrocytes results in increased levels of GFAP, a class-III intermediate filament [4]. By proteomic analysis, GFAP appeared as one of the main carbonylated proteins, especially in HD striatum [22]. Because the increased carbonyl levels of GFAP presented in HD could reflect the increased amount of protein present, a deeper analysis of the GFAP carbonylation was performed using narrow immobilized pH gradient strip [22]. In a 2D gel, GFAP appeared in more than 20 spots with different Mw (from 35 to 50 kDa) and isoelectric points (from 4 to 5.5). When GFAP carbonylation and protein expression were normalized, it was inferred that, on average, GFAP was approximately twice as oxidized in striatum from HD patients than in that of matched controls. In the cortex, differences between HD and controls were not clear. However, specific carbonylation of some of the multiple existing isoforms cannot be ruled out. It is worth mentioning that GFAP has been identified as a major target of oxidative damage in Pick disease [103], Alzheimer disease [104] and aceruloplasminemia brain [105]. Whether this elevated sensitivity to oxidation is associated with loss of function and the potential consequences for cell physiology remains to be answered.

### 3.6 Transitional endoplasmic reticulum ATPase

Transitional endoplasmic reticulum ATPase, also known as valosin-containing protein (VCP), is a member of the AAA family of ATPases associated with several cellular activities. Mutations in VCP cause inclusion body myopathy (IBM), Paget disease of the bone, and frontotemporal dementia (IBMPFD). The role of VCP in autophagy has been demonstrated in IBMPFD, and

impaired autophagy due to VCP dysfunction explains the pathology seen in this disease, including abnormal TDP-43 (a DNA-binding protein) accumulation in neuronal and muscle inclusions [106]. In HD, VCP colocalizes with protein aggregates in neurons of HD patients and in cultured cells expressing diseased proteins [107]. TDP-43 also accumulates in the brain of HD cases, although the mechanism of this alteration is not known [108]. By proteomic analysis, VCP appears as an oxidatively modified protein in HD. Oxidative stress has been described to inactivate VCP ATPase activity [109]. Moreover, accumulation of protein aggregates might result in VCP dysfunction, causing impairment of the ubiquitin-dependent protein degradation, ER stress, oxidative stress and pathological involvement of mitochondria [110]. Thus, in HD, the observed VCP carbonylation in striatum may result in loss of its activity affecting the above mentioned processes, including autophagy, and may play a role in abnormal TDP-43 inclusions in HD.

### 3.7 Dimethylarginine dimethylaminohydrolase 1

Dimethylarginine dimethylaminohydrolase 1 (DDAH1) hydrolyzes both N(G),N(G)-dimethyl-L-arginine (ADMA) and N(G)-monomethyl-L-arginine, both of which act as inhibitors of NOS. DDAH1 has, therefore, a role in nitric oxide generation. DDAH1 is highly resistant to high doses of H<sub>2</sub>O<sub>2</sub> but is inhibited upon specific Cys-S-nitrosylation by S-nitroso-L-homocysteine [111]. Moreover, L-homocysteine inhibits DDAH1 activity by reacting with one Cys at the active site [112], causing ADMA accumulation and decreasing nitric oxide production and bioavailability. DDAH1 is also inhibited by pathophysiologic concentrations of the lipid peroxidation product 4-HNE [113]. The mechanism of inhibition is related to the formation of Michael adducts on a catalytic His.

In general, it is accepted that nitric oxide might play a toxic role in HD; however, the exact function is still uncertain. In R6/1 mice, NOS expression was increased at 19 weeks, followed by

a decreased level in 35-week-old mice, compared with controls, a phenomenon that parallels the changes in NOS enzyme activity [114]. This seems to indicate that, at least in R6/1 mice, nitric oxide is involved in the onset of the progressive neurological phenotype, but its role can change at different stages of the disease. Perhaps oxidation of DDAH1 found in human HD post-mortem samples, which could negatively affect nNOS activity, resembles the final stage of the disease in the transgenic mice.

### 3.8 Voltage-dependent anion channel 1

The voltage-dependent anion channel 1 (VDAC1) located in the outer mitochondrial membrane acts as a gatekeeper for the entry and exit of mitochondrial metabolites. VDAC, together with the ADP/ATP translocase and cyclophilin-D, form the mitochondrial permeability transition pore (PTP) allowing cross-talk between mitochondria and the cytosol [115]. Carbonylation of VDAC was described in R6/2 transgenic mice compared to control littermates [45]. Although the effect of such oxidative modification was not explored, the authors suggested that impairment of VDAC1 can explain the calcium overload and PTP-induced mitochondrial dysfunction observed in HD mouse models and human brain [116]. Also, VDAC oxidation might affect its functional properties, thus contributing to disruption of PTP permeability. This would lead to a lower  $\text{Ca}^{2+}$  threshold for depolarization, to a decreased membrane potential, and ultimately to alteration of mitochondrial permeability. Changes in cytochrome c release and apoptosis would be among the consequences. Consistent with this notion, cyclosporin A, an inhibitor of PTP, avoids the toxic effects of 3-NP in vivo and in vitro, presumably by blocking the pro-apoptotic release of cytochrome c [117].

## 4. Selectivity of protein oxidation

An important aspect to consider is why the proteins listed in Table 1 are especially prone to suffer oxidative damage. What do all these identified targets have in common? The most important shared trait, which affects not all but most of them, is that they bind to molecules such as iron or nucleotides phosphate like ATP/ADP. Aconitase and cytochrome b-c1 (complex III) are proteins which bind iron directly or indirectly. Pyruvate kinase, citrate synthase, aconitase, cytochrome b-c1 complex, ATP synthase, creatine kinase, Hsc71, TRiC, Hsp90, pyridoxal kinase and VCP are nucleotide-binding proteins. It should be kept in mind that all nucleotide phosphates like ATP and ADP have a coordinated magnesium ion. It has been proposed that under oxidative stress and iron overload, both observed in HD, nucleotide-binding proteins are specific targets of oxidative damage [41]. This sensitivity has been attributed to the ability of Fe(II) to replace magnesium ions bound to the phosphate groups of the nucleotides; their reaction with H<sub>2</sub>O<sub>2</sub> or oxygen would then trigger the formation of ROS. As an example, oxidation of ATP synthase in vitro has been shown to be mediated by iron binding to magnesium-binding sites [118]. In this context, enzymes with a Fe-binding site or prosthetic groups with iron are especially prone to suffer oxidative damage [119]. It is well known that aconitase is especially sensitive to oxidative stress. Aconitase loses its Fe-S cluster under oxidative stress conditions and is carbonylated under different conditions and pathologies [46]. A similar situation could occur in the cytochrome b-c1, which has a heme-Fe group. In addition to binding to specific nucleotides, carbonylation of Hsc71, TRiC, and Hsp90 also can be explained by their interaction with damaged proteins produced under oxidative stress. It has been suggested that oxidized proteins contain reactive species that can, in turn, damage other proteins [120].

A second group of proteins (GAPDH, Prx1, Prx6, mHtt, DDAH1) seen as oxidized in HD presents highly reactive thiol -or His- groups at or near their active site. Such Cys have been described to be easily oxidizable, producing oxidized reversible forms of thiol, like inter/intra disulfides bonds in the case of mHtt, or irreversible forms like cysteic acid (also known as sulfonic

acid (Cys-SO<sub>3</sub>H) in the case of Prx1 and Prx6. DDAH1 also has a reactive Cys which has been described to be nitrosylated, but the presence of a carbonyl group in this protein can be due to reaction with HNE (Michael addition) on a catalytic His [113]. However, HNE is more prone to react to thiol groups [121] and because it is well known that GAPDH has a highly reactive Cys in its active site, HNE binding could explain its carbonylation. In two cases (enolase and VDAC1) their carbonylation remains to be explained, although oxidative modification of enolase has been described in several stress conditions [46].

## 5. Concluding remarks

The existence of oxidative stress in affected areas of HD patients and animal models is widely assumed. Although it is not completely understood how mHtt causes this stress, a toxic gain of function is well accepted. It is clear that mHtt expression results in increased levels of oxidatively damaged proteins and other macromolecules in specific areas of the HD-affected brain. Fig. 2 summarizes the altered functions and metabolic pathways affected by ROS production as a consequence of mHtt. Oxidation of some specific proteins would, in turn, increase the ROS levels. This produces a vicious cycle with a progressively increased stress situation that, in addition to the oxidation of several chaperones and decreased proteasomal function, might contribute to the accumulation of damaged proteins. An important question still under discussion is whether oxidative stress and its consequences, such as protein (but also DNA and lipid) damage, play a pivotal role in the pathogenesis of HD or are a secondary consequence of striatal degeneration. From our point of view, research results increasingly point to the important role of oxidation of some proteins, even in the early stages of the disease, particularly in impairment of energy production and protein folding. Although in past studies treatment with antioxidants failed to deliver clear benefits to HD patients, increased doses of coenzyme Q and creatine are currently under evaluation in phase III clinical trials.

Results from our group indicate a possible important role of vitamin B6 metabolism. Decreased levels of PLP would affect important pathways known to be altered in HD, including synthesis of several neurotransmitters, GSH and the kynurenine pathway. In this context, treatment with PLP, the active form of vitamin B6 (instead of pyridoxine or piridoxal), should be considered for evaluation as a therapy.

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**Table 1**  
**Protein targets of oxidative damage in HD**

Protein	Source	Observed modification	Oxidation effect	Reference
<b>Energy metabolism</b>				
$\alpha$ -Enolase	Human striatum R6/2 mouse	Carbonylation Carbonylation	Unknown Inactivation	[37] [45]
$\gamma$ -Enolase (brain-specific isoform)	Human striatum R6/2 mouse	Carbonylation Carbonylation	Unknown Inactivation	[22] [45]
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Human striatum	Carbonylation	No inactivation	[37]
Pyruvate kinase isoenzymes M1/M2 (PK)	Human striatum	Carbonylation	No inactivation	[37]
Citrate synthase, mitochondrial (CS)	Human striatum	Carbonylation	Inactivation	[37]
Aconitase	Human striatum R6/2 mouse	Carbonylation Carbonylation	Inactivation Inactivation	[22] [45]
Cytochrome b-c1 complex subunit 2, mitochondrial	Human striatum	Carbonylation	Unknown	[37]
ATP synthase subunit alpha	Human striatum	Carbonylation	Inactivation	[37]
Creatine kinase	Human striatum R6/2 mouse	Carbonylation Carbonylation	Inactivation Inactivation	[22,37] [45]
<b>Oxidative stress defense and folding</b>				
Heat shock cognate 71 kDa protein (Hsc71)	Human striatum	Carbonylation	Unknown	[37]
T-complex protein 1 subunit beta (TCP1, TRIC)	Human striatum	Carbonylation	Unknown	[37]

Heat Shock Protein 90 (Hsp90)	R6/2 mouse	Carbonylation	Unknown	[45]
Peroxiredoxin 1 (Prx1)	Human striatum	Acidic pl shift (putative cysteic acid formation)	Unknown (putative inactivation)	[22]
Peroxiredoxin 6 (Prx6)	Human striatum	Acidic pl shift (putative cysteic acid formation)	Unknown (putative inactivation)	[22]
<b>Pyridoxal phosphate metabolism</b>				
Pyridoxal kinase	Human striatum	Carbonylation	Unknown (putative degradation)	[37]
	Tet/HD94 mouse	Carbonylation	Unknown (putative degradation)	
$\alpha$ -Aminoadipic semialdehyde dehydrogenase (antiquitin 1)	Human striatum	Carbonylation	Unknown	[37]
<b>Miscellaneous</b>				
Mutant Huntingtin	CAG140 mice and PolyQ transfected COS cells	Cys oxidation	Intermolecular disulfides (stable oligomerization)	[11]
Glial fibrillary acidic protein (GFAP)	Human striatum	Carbonylation	Unknown	[22]
Transitional endoplasmic reticulum ATPase (VCP)	Human striatum	Carbonylation	Unknown	[37]
Voltage-dependent anion channel 1 (VDAC)	R6/2 mouse	Carbonylation	Unknown	[45]
N(G), N(G)-dimethylarginine dimethylaminohydrolase 1 (DDAH1)	Human striatum	Carbonylation	Unknown	[37]

## Figure legends

**Fig. 1.** Oxidation of pyridoxal kinase (PDXK) and antiquitin 1, involved in pyridoxal-5-phosphate (PLP) metabolism, may impair synthesis of neurotransmitters and glutathione (GSH). All aminotransferases and the kynurenine pathway would also be affected. CS, cystationine  $\beta$ -synthase, CL, cystationine  $\gamma$ -ligase; GAD, glutamate decarboxylase; 5-HTP, 5-hydroxytryptophan; 5-HTP DC, 5-hydroxytryptophan decarboxylase; DOPA DC, dopamine decarboxylase; 3-HK, 3-hydroxyquinurenine; KMO, kynurenine monooxygenase; KYNT, kynurenine transaminase; P6C, L- $\gamma$ -piperidine-6-carboxylate; PNPOX, pyridoxamine/pyridoxine 5-phosphate oxidase; QUIN, quinolinic acid; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase.

**Fig. 2.** Oxidative damage of target proteins in HD might lead to dysfunction of multiple processes inside the cell. ADMA, dimethyl arginine; CK, creatine kinase; CS, citrate synthase; CS, cystationine  $\beta$ -synthase, CL, cystationine  $\gamma$ -ligase; DDAH1, dimethylarginine dimethylaminohydrolase 1; DMA, dimethylamine; DOPA DC, dopamine decarboxylase; GAD, glutamate decarboxylase; GAPDH, glyceraldehyde 3-phosphate; dehydrogenase; GFAP, glial fibrillary acidic protein; Hsc71, heat shock cognate 71; Hsp90, heat shock protein 90; 3-HK, 3-hydroxyquinurenine; 5-HTP DC, 5-hydroxytryptophan decarboxylase; KYNT, kynurenine transaminase; PDXK, pyridoxal kinase; nNOS, neuronal nitric oxide synthase; PK, pyruvate kinase; Prx1/6, peroxiredoxin 1/6; TRiC, TCP-1 ring complex; UPS, ubiquitin-proteasome system; VCP, valosin-containing protein.